Optimization of the Sliced Testis Steroidogenesis Assay

Draft Part I Letter Report

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1.0 INTRODUCTION

1.1 Background

In 1996, the Food Quality Protection Act (FQPA) amendments were enacted by Congress to authorize the Environmental Protection Agency (EPA) to implement an Endocrine Disruptor Screening Program (EDSP) on pesticides and other substances found in food or water sources for endocrine effects in humans (FQPA, 1996). In this program, comprehensive toxicological and ecotoxicological screens and tests are being developed for identifying and characterizing the endocrine effects of various environmental contaminants, industrial substances, and pesticides. A two-tiered approach will be utilized. Tier 1 employs a combination of *in vivo* and *in vitro* screens, and Tier 2 involves *in vivo* testing methods using two-generation reproductive studies. A steroidogenesis assay is proposed as one of the Tier 1 screening battery assays.

A detailed review paper (DRP) about steroidogenesis was prepared. The DRP (1) summarized the state of the science of the *in vivo, ex vivo,* and *in vitro* methodologies available for measuring gonadal steroidogenesis; (2) for each methodology, presented a review of the individual assays and representative data generated by investigators who used the assay to evaluate a substance for steroidogenic-altering activity; (3) provided an evaluation of the various methodologies and the assays as tools for screening substances with suspected steroidogenic activity; (4) recommended a particular screening method and assay as a screening tool; and (5) described the strengths, weaknesses, and implications for further research associated with the recommended screening assay.

The *in vitro* sliced testis steroidogenesis assay was selected as the most promising screening tool for identifying substances with steroidogenic-altering activity. The sliced testis assay was recommended because it can be conducted at a minimal cost, quickly, and simply with standard laboratory equipment and basic laboratory training; the preparation is stable and the parenchyma remains viable over a sufficient time period to measure changes in end-product hormone production; the assay is relatively sensitive and specific; the assay uses parenchyma that maintains the cytoarchitecture of the organ; the assay uses a reduced number of animals (up to quartered testis slices); the assay should be relatively easy to standardize (by optimization); and the assay has a well-defined endpoint in testosterone and, if desired, can be modified to include additional intermediate hormonal endpoints.

Although a promising tool, the sliced testis assay remains to be fully tested as an assay that can meet all the demands of an endocrine disruptor screening tool. Concerns raised by the EPA and Endocrine Disruptor Methods Validation Subcommittee (EDMVS) suggested that experiments be conducted to ensure the optimization of the assay prior to more rigorous pre-validation and validation testing. The most notable concerns were associated with 1) various incubation variables, 2) variables that

affect optimal human chorionic gonadotrophin (hCG) stimulation, 3) characterization of the parenchymal post-slicing equilibration time, and 4) parenchymal viability. In addition to these most notable concerns, other factors that could potentially affect the optimal performance of this assay were identified. The objective of this optimization was to describe in detail the experiments designed to provide data for setting in place the procedures and parameters that will optimize the performance of this assay.

1.2 Objectives

The study plan for testing the factors described in the previous section involved two phases and utilized single factor and factorial experimental designs. A diagram of the experimental design for this study is illustrated in Figure 1.

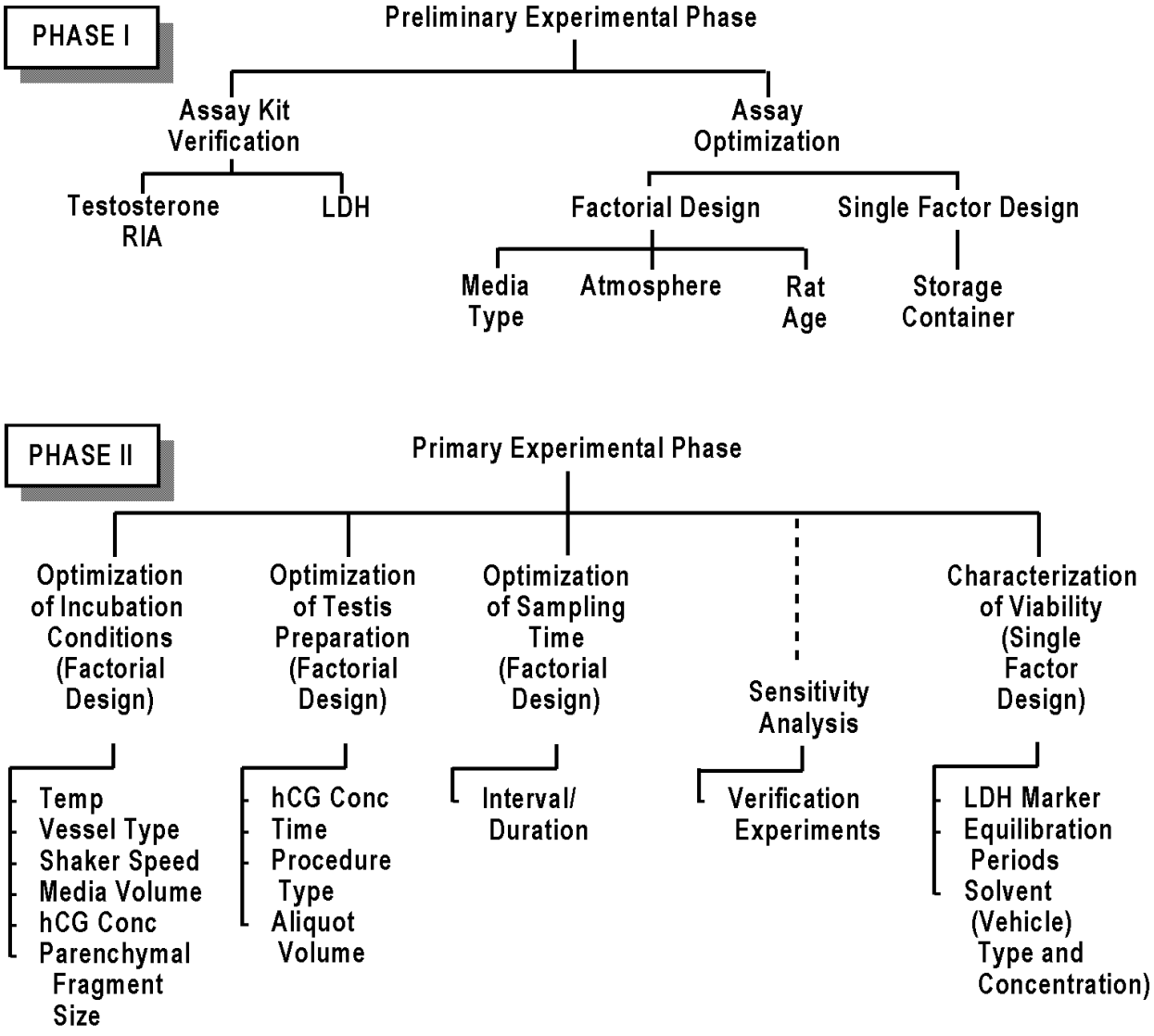

Figure 1. Sliced Testis Steroidogenesis Assay Experimental Design Organizational Diagram

The study was divided into Phases I and II.

In Phase I, the Preliminary Experimental Phase, the analytical assays planned for use were verified, storage containers and lengths of storage were selected and three factors that may affect the

performance of the assay were tested. The reasoning for including these three factors in the preliminary phase was to establish early whether a given level of each factor was going to affect assay performance. Although any factor listed in the study plan could have been rationalized to fit such a criteria, inclusion in the preliminary phase also required that the factor be unlikely to have an interaction, or at best a minimal interaction, with another experimental factor. Although subjective, these factors were believed to best fit these criteria. Furthermore, it was believed essential to establish the optimal level for each of these factors before proceeding with the factorial experiments since an effect of one of these would require additional verification experiments after sensitivity analysis. Finally, by establishing the media type early on in the experiment, the analytical assay verification testing (Phase I) and Optimization of Sample Testing (Phase II) could be initiated earlier in the study milestone schedule.

2.0 MATERIALS AND CHEMICALS

2.1 Reagents andSolutions

No test substances were used in this study. The chemicals used in this study were used to prepare reagents and solutions for the assay. All reagents and solutions had appropriate information documented, which included the identity, concentration, storage requirements, and expiration date. Reagents and solutions were prepared according to Standard Operating Procedures.

2.2 Standards

Verification of the analytical assays required testosterone for the radioimmunoassay (RIA) method and LDH for the spectrophotometric assay. In addition, hCG was used as a stimulant of the sliced testis bioassay. These substances were considered standards.

2.2.1 Testosterone

Chemical Name: Testosterone
CAS No.:58220
Molecular Weight:288.4
Solubility: Clear colorless to very faint yellow solution at 100 mg/mL in chloroform
Supplier: Sigma-Aldrich Chemical Company
Lot No.: 6384-70-8
Purity:NLT 98%
Storage Conditions: 2 Year shelf life

A safety protocol exists for the use of the radioactive form of testosterone.

2.2.2 Human Chorionic Gonadotropin (hCG)

Chemical Name: hCG
CAS No.: 9002-61-3
Molecular Weight: 36,700
Solubility: H_2O
Supplier: Calbiochem
Lot No.: B11174, B51120
Purity: approx. 30% hCG by weight
Storage Conditions: Freezer (-20°C). Following reconstitution, aliquot and freeze (-20°C). Stable for 2 years as supplied.

2.2.3 LDH

Chemical Name: Lactate Dehydrogenase
CAS No.: EC 1.1.127
Source: Rabbit muscle
Solubility:
Supplier: Sigma-Aldrich Chemical Company
Lot No.: 99H7480
Purity: NLT %
Storage Conditions: 2-8°C Year shelf life

3.0 METHOD FOR PROTOTYPICAL ASSAY

The prototypical assay describes the sliced testis assay using the conditions that are believed to be the starting conditions of the assay. This section does not describe any experiments to be conducted; rather, it describes the settings of all factors, except for the one that is being tested in order to perform the optimization experiments described in the following sections. A run is defined as a single sample vessel with assay components.

The sliced testis assay prototype uses a 15 week old male Sprague-Dawley rat, which is euthanized and its testes removed. The testes are decapsulated, weighed, and placed in cold (4°C) DPBS. The media is medium-199 (Gibco) that has added 0.71 g sodium bicarbonate, 2.1 g HEPES, 1.0 g/L BSA, and 0.025 g/L soybean trypsin inhibitor, and is adjusted to a pH of 7.4. The time from removal to the time of slicing is held to under 1 hour. Each testis is sliced along the longitudinal axis into approximately 4 slices. Each slice is placed in a 20 mL borosilicate scintillation vial (loosely capped) that contains 5 mL of media (Figure 2). The vials containing the testicular sections and media are incubated at

34°C on a shaker (at 135 rpm) in 5 percent CO_2/95 percent air. After the first period of incubation, the media is removed and discarded. Fresh media (5 mL) is added to the vial and an aliquot of media (0.5 mL) is collected. The sample is centrifuged and the supernatant transferred to a labeled vial and stored at approximately -70°C in a siliconized plastic container for no more than one month. This sample is the baseline sample. Next, one half of the vials are challenged with a stimulant, e.g., hCG, and the other half are not. The final hCG concentration is 0.1 IU/mL. Additional media samples are collected from the vials at 1, 2, 3, and 4 hours post-challenge. These media samples are also stored frozen for later analysis. Samples are analyzed for testosterone using an RIA method. All samples for a given day's set of runs should be analyzed in the same testosterone RIA.

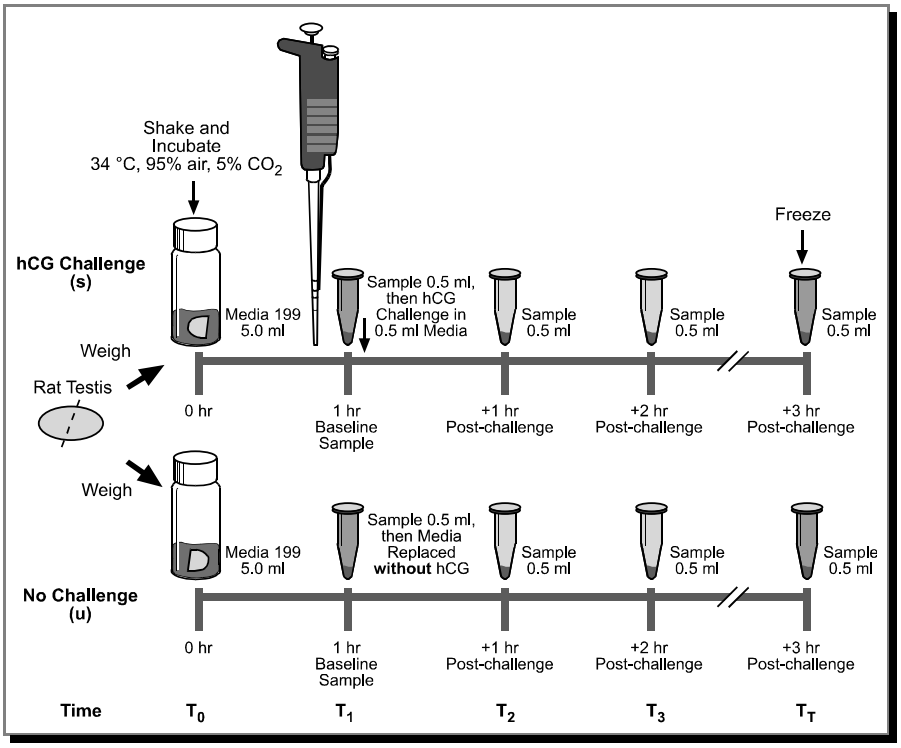

Figure 2. Technical Flow Illustration of the sliced testis steroidogenesis assay

4.0 METHODS FOR PHASE I - PRELIMINARY EXPERIMENTAL PHASE

Phase I is comprised of the verification experiments for the two analytical assay methods (testosterone RIA and LDH spectrophotometry), the determination of the preferred storage container and storage length, and the optimization experiments for the three factors to be tested (media type, incubation atmosphere, and animal age). The testosterone RIA method was verified prior to conducting any optimization assay since it is was needed as an endpoint to determine optimization of the sliced testes assay. The storage container determination was also performed before any further optimization assays were run. The determination was made using standards of testosterone in the prototypical media, modified M-199 without phenol red.

4.1 Testosterone Radioimmunoassay

The objective of this experiment was to verify that testosterone can be measured in the sliced testis assay media. A RIA commercial kit (Diagnostic Products Corporation, Los Angeles, CA), that utilizes ^{125}I-testosterone and a testosterone-specific antibody affixed to polypropylene culture tubes, was used to measure testosterone. The assay was verified in all three of the potential assay media.

Testosterone (Sigma, St. Louis, MO; T-1500) was used for preparing the standard curve and was stored desiccated at room temperature in the RTI vault. A standard was prepared in ethanol (0.1 mg/ml). Up to an 8-point standard curve but not less than a 4-point standard curve was prepared using standards with concentrations of 0.07, 0.16, 0.41, 1.02 , 2.56, 6.4, 16, and 40 ng/ml in PBS-Gel Buffer (0.1 M phosphate buffered saline with 0.1% (w/v) sodium azide and 0.1% (w/v) gelatin, pH 7.4). In addition, procedural controls were included in each run. The standard curve points and the procedural controls were prepared in quadruplicate; the bioassay unknowns and the internal standards (see below) were prepared in duplicate. The volume of all standards and controls (including bioassay unknowns) were adjusted to 50 µL by adding the PBS-Gel Buffer. Next, 1 ml ^{125}I-testosterone was added to each antibody-coated tube and mixed (Vortex). The tubes were incubated in a 37˚C water bath for three hours, during which time testosterone, whether labeled or unlabeled, competed for testosterone specific antibody binding sites. At the end of the incubation period, the free (unbound) testosterone, in the supernatant fluid of all tubes was aspirated and tubes were wiped clean of fluid. The bound testosterone was counted in a gamma counter for 1 minute. The concentration of testosterone was estimated against the standard curve. Values were reported as a mean concentration (ng/mL) of duplicate analyses, unless only a single value was available. Verification of the testosterone assay involved preparation of internal standards (at least three) using spiked media with concentrations ranging from 12.5 to 500 ng/mL. Each concentration was run at each of three volumes - 10, 25, and 50 µL, to check for parallelism, and each sample was adjusted to 50 µL by adding the PBS-Gel Buffer. The low and high standards were analyzed in at least duplicate. Verification was based on results determined for accuracy, precision, specificity, and linearity. Accuracy is expressed as the relative error, which was determined by comparing the measured to the target concentration. Relative errors within 15 percent were acceptable. Precision is expressed as the

relative standard deviation (RSD) or coefficient of variance (CV), which was determined by calculating the mean and standard deviation (sd) of the low and high standards. A RSD or CV within 15 percent was acceptable. The sensitivity was acceptable if the means of the blanks and low standards were significantly different at the 5 percent significance level. Linear determinations of the standard curve line were made and a correlation coefficient (r) calculated. An r of 0.90 or greater was considered acceptable.

Inter- and intra-assay variability was determined. The intra-assay variability was determined from the precision results calculated from the results obtained by measuring the low and high standards in triplicate. The inter-assay variability was determined by repeated analyses of the standards by generating a standard curve on three different days.

4.2 Lactate Dehydrogenase Spectrophotometric Assay

The objective of this experiment was to verify that LDH can be measured in the sliced testis assay media. The LDH assay measures the rate at which NADH is formed when NAD is reduced when it catalyzes the oxidation of lactate to pyruvate. NADH is measured at 340nm using a kinetic-spectrophotometric method. The assay and samples are temperature sensitive. The samples should not be refrigerated or frozen. The assay has been characterized for assay conditions at 37^{o}C. LDH activity is expressed in U/L.

Qualification of the assay using Media 199 without phenol red consisted of determination of sensitivity and dilutional linearity. These were the only qualification tests specific to media use. Intra-assay imprecision and inter-assay imprecision were based on serum based controls normally used for instrument monitoring of quality control. Accuracy was based on the linear regression of a media sample spiked with purified LDH and diluted with media.

Intra-assay imprecision was measured by assaying ten quality control samples within a single run. The mean standard deviation (SD) and coefficient of variation (CV) was calculated. The CV should be less than 10%. Inter-assay imprecision was measured by assaying ten quality control samples over ten separate runs. The mean standard deviation (SD) and coefficient of variation (CV) was calculated. The CV should be less than 10%.

Sensitivity of the assay or limit of detection was determined by analysis of twenty samples of media with no LDH present in the media. A low level of control was also analyzed to determine the lowest level of detection. Two standard deviations from the mean of the media activity was used as the limit of detection.

Dilutional linearity was used to determine accuracy and linearity limits. A sample of media was spiked with purified LDH (Sigma #L1254). This was diluted with Media 199 without phenol red to within

the linear limits of the assay. A series of dilutions by serial dilution of the spiked sample was conducted in triplicate. The linear regression of the line compared to the theoretical activity should be between 0.990 and 1.100.

All calculations were performed using EP Evaluator, release 3.0 statistical analysis software from David Rhoads Associates, Inc., Kennett Square, PA.

4.3 Phase 1 Optimization Experiments for Media Type, Gaseous Atmosphere, Rat Age, and Storage Container Type

4.3.1 Media Type

The objective was to determine the effect of different types of media (with specified components) on testosterone production using the sliced testis assay. The prototypical assay conditions were used except with regard to the types of media.

The media types tested were:

RPMI-1640 media (without phenol red), 10% FCS, 50 ug/mL soybean trypsin inhibitor

Medium-199 (Gibco), 0.71 g Na bicarbonate, 2.1 g HEPES, 1.0 g/L BSA, 0.025 g/L soybean trypsin inhibitor, adjusted to pH 7.4

Eagles MEM

4.3.2 Gaseous Atmosphere

The objective was to determine the effect of different types of gaseous atmospheres on testosterone production using the sliced testis assay. The prototypical assay conditions were used except with regard to the gaseous atmosphere.

The atmospheres tested were:

5% CO_2/95% air

5% CO_2/95% O_2

air (three gases).

4.3.3 Rat Age

The objective will be to determine the effect of age on testosterone production using the sliced testis assay. The prototypical assay conditions will be used except with regard to the age of the rat used to obtain the testes.

The ages to be tested are:

11 weeks of age

15 weeks of age

22 weeks of age.

4.3.4 Storage Container Type

The objective was to determine the effect of storage type containers on the stability of testosterone in media. The types of containers tested were: siliconized plastic and non-siliconized plastic.

4.4 Phase 1 Experimental Design

4.4.1 Factorial Design Experiments

These experiments were conducted as a 3^3 full factorial design with one replicate per condition. The experimental factor levels are summarized in Table 2. The factorial test conditions are displayed in Table 3. The 27 factor level combinations were run in random order. Each combination was run with and without hCG stimulation, for a total of 54 test runs. For each test run responses (ng T/ml) were determined at 1, 2, 3, and 4 hours after media refreshment.

Table 1. Summary of Experimental Factors for Phase 1 Optimization

Factor Identification	Units	Experimental Levels			Coded Experimental Levels		
		1	2	3	1	2	3
Media Type	NA	RPMI-1640	medium-199	Eagles-MEM	-1	0	+1
Gaseous Atmosphere	NA	5% CO_2 / 95% air	5% CO_2 / 95% O_2	air	-1	0	+1
Rat Age	wks	11	15	22	-1	0	+1

NA = not applicable.

Table 2. Factorial Test Conditions for Phase 1 Optimization Experiment

Media Type	Gaseous Atmosphere	Rat Age
-1	-1	-1
-1	-1	0
-1	-1	+1
-1	0	-1
-1	0	0
-1	0	+1
-1	+1	-1
-1	+1	0
-1	+1	+1
0	-1	-1
0	-1	0
0	-1	+1
0	0	-1
0	0	0
0	0	+1
0	+1	-1
0	+1	0
0	+1	+1
+1	-1	-1
+1	-1	0
+1	-1	+1
+1	0	-1
+1	0	0
+1	0	+1
+1	+1	-1
+1	+1	0
+1	+1	+1

4.4.2 Phase 1 Single Factor Experimental Design

The objective of this set of experiments was to determine the effect of storage container type on the stability of testosterone in the media. This experimental series did not use the sliced testis assay. Stability was assessed as a function of sample handling factors. The incubation medium that will be used will be determined in the factorial experiments (Phase I). To conduct the stability experiments, a known amount of testosterone was added to the media to achieve a specified target concentration. The target concentration was determined from the media type experiments in Phase I and was in the range of the lowest testosterone concentrations measured in the sliced testis assay. Using this target concentration, the measured concentration was compared to the target concentration. The stability was evaluated based on the difference between the measured and target concentrations. If no statistical difference existed at the 5 percent level, then the sample was determined to be stable under the conditions tested.

4.5 PHASE I DATA EVALUATION

Upon completion of the Phase I optimization experiments, the results were reviewed for a possible change in the prototype conditions with regard to the four factors tested. A decision was made as to whether the Phase II optimization experiments would be conducted using the original prototype or modifications made to the media type, atmosphere, age of rat and/or storage container type used for the remaining experiments. The endpoint was the amount of testosterone released into the media during the sliced testis assay. The test conditions resulting in the highest values for testosterone were used in Phase II.

5.0 RESULTS AND STATISTICAL ANALYSES

5.1 Testosterone RIA Verification

The Testosterone RIA kit from Diagnostic Products was used to verify that the M-199 media without phenol red could be used for the sliced testis assay and provide accurate results for the RIA assay.

Table 3. Values of Standards on Testosterone RIA		
Value of Standard	**Factor**	**Reading**
8 ng/ml	1	9.33
8 ng/ml	1	8.70
8 ng/ml	1	9.19
8 ng/ml	1	8.86
8 ng/ml	1	9.35
8 ng/ml	2	9.63
8 ng/ml	2	10.16
8 ng/ml	2	10.54
8 ng/ml	2	11.08
8 ng/ml	2	11.86
8 ng/ml	5	10.96
8 ng/ml	5	11.60
8 ng/ml	5	12.41
8 ng/ml	5	11.56
8 ng/ml	5	13.03
2 ng/ml	1	2.56
2 ng/ml	1	2.77
2 ng/ml	1	2.56
2 ng/ml	1	2.59
2 ng/ml	1	2.46
0.5 ng/ml	1	0.63
0.5ng/ml	1	0.80
0.5ng/ml	1	0.76
0.5ng/ml	1	0.74
0.5ng/ml	1	0.71
Unspiked Media		Below Detection Limits of 0.04ng/mL

Table 4. Testosterone RIA Intra-assay CV				
	Number	**50 µl**	**25 µl**	**10 µl**
Unspiked M199	2	Blanks		
+ 8 ng/ml	10	5.24%	8.64%	7.68%
+ 2 ng/ml	10	6.09%		
+ 0.5 ng/ml	10	13.34%		

Table 5. Testosterone RIA Percent Recovery			
	50 µl	**25 µl**	**10 µl**
+ 8 ng/ml	113.8	133.3	149.0
+ 2 ng/ml	129.5		
0.5 ng/ml	146.5		

Table 6. Testosterone RIA Parallelism			
	50 µl	**25 µl**	**10 µl**
+ 8 ng/ml	9.10 ng/ml	10.66 ng/ml	11.92 ng/ml

The Index between 50 and 25 10 µl was 117.1%, between 25 and 10 µl was 111.8% and between 50 and 10 µl was 130.99%.

5.2 LDH Verification

See Appendix A

Statistical Analysis of the Phase I Assay Optimization Experiment

Objectives

The assay optimization experiment involves three factors (media type, atmosphere, and rat age) which are run in a 3^3 factorial arrangement; each of these 27 trials is run with and without hCG stimulation and repeated measurements are taken at baseline (time 0) and at 1, 2, 3, and 4 hours after baseline. The conditions are identified in Table 7. Objectives of the experiment are:

1. To determine the set of conditions that yields the highest estimated testosterone level, and
2. To determine the set of conditions that yields the largest with-versus-without-hCG difference in testosterone levels.

Data

Two basic SAS data sets were constructed from the raw data and two fundamental types of dependent variables were used in the analyses of each type:

Date Set 1: Cases without hCG stimulation

Dependent variables: testosterone concentrations

Dependent variables: (natural) logarithm of testosterone concentrations

Data Set 2: Cases with hCG stimulation

Dependent variables: testosterone concentrations

Dependent variables: (natural) logarithm of testosterone concentrations

Each data set can be viewed as consisting of 27 observations (rows). Each observation includes dependent variable values for 4 time points and a corresponding baseline level. Each observation also includes data identifying the levels of the pertinent factors. Data are listed in Table 8 for the unchallenged samples, and in Table 9, for the challenged cases.

Statistical Analysis Methods

Objective 1. Several statistical analysis methods were used to address the first objective. Analysis of variance (ANOVA) and analysis of covariance (ANOCOVA) were used to analyze the data for each individual time point (including the baseline) and a mixed-model ANOCOVA method was used to jointly analyze the data (across time points 1 through 4). The ANOCOVA models utilized the baseline level (or log-level) as a covariate. For each type of analysis, all main effects and two-factor interactions

(2fi) of the three factors were initially included in the models. Tests for interactions were conducted and where they were not detected as statistically significant (p=0.05), a reduced model was employed that retained the main effects, the baseline covariate (where applicable), and only those 2fi deemed to have significant effects. Additional details are provided in the Results section.

Objective 2. For each of the 27 trials, differences between the with-hCG and the without-hCG testosterone levels were computed for each hour (including baseline). These differences were computed on both the original and log scales. Analysis of variance (ANOVA) was used to analyze these differences for each individual time point (including the baseline). For each model and type of data, all main effects and 2fi of the three factors were initially included in the models. Tests for interactions were conducted and where they were not detected as statistically significant (p=0.05), a reduced model was employed that retained the main effects and only those 2fi deemed to have significant effects. Additional details are provided in the Results section.

Results

Overall Characterization of the Data. Table 10 provides summary statistics characterizing the testosterone levels in the non-hCG-stimulated data set. This summary ignores the particular experimental factors. The top portion of the table gives, by hour, the sample size (n), the mean, standard deviation, sum, minimum, and maximum. These variables are denoted as yJ, where J denotes the hour and takes on values of 0, 1, 2, 3, and 4. The lower portion of the table gives the correlations between the hourly data. The following trends are apparent:

- the means continue to increase over time
- the standard deviations also increase over time (i.e., as the mean level gets larger)
- the correlations are generally high, as tend to be largest for adjacent hours.

Table 11 provides a similar summary for the log-scaled data; these variables are denoted as lyJ, where J denotes the hour. Similar trends for the means and correlations are evident, but the standard deviations tend to be fairly stable across the various time points.

Tables 12 and 13 furnish comparable information for the hCG-stimulated samples. Similar trends are evident for these data. Mean levels tend to be much higher than for the non-stimulated samples.

Analysis of Baseline Data. Since we intend to adjust for baseline (time 0) levels for subsequent analyses of the hourly (non-baseline) data, it is important to understand how the experimental factors affect the baseline levels. For instance, if one of the factors does impact the baseline levels, then adjusting for baseline levels in those subsequent analyses may obscure the effect of the experimental factor. Table14 presents the results that summarize the ANOVA results for the baseline data. Initially, we fit an ANOVA model that included all mean effects (denoted as z1, z2, and z3) and all two-factor

interactions (denoted as z1*z2, z1*z3, and z2*z3). We examined the statistical significance of each of the interactions and reduced the model to contain only main effects and the pertinent 2fi. For three of the four cases considered, only the main effects were retained; for the log-scale, without hCG case, two of the interaction effects were deemed significant. Among the three experimental factors, it is clear that the rat age (z3) has the most pronounced effect on the baseline levels (estimated testosterone levels are 0.54, 0.58, and 0.17 for 11-week, 15-week, and 22-week old animals). For the non-hCG-stimulated case, media type also impacted the baseline levels with the highest level occurring for the z1=0 case (0.54 versus 0.38 for the other two media). The lower portion of Table 14 furnishes statistics characterizing the model fit:

R^2 = the proportion of variability accounted for by the model,
RMSE = root mean squared error = the square root of the residual variance,
C.V. = the coefficient of variation = the RMSE divided by the mean testosterone level (times 100%).

Analyses Directed at Objective 1. Two fundamental types of statistical analysis were used to address objective 1 (assessing the effects of the experimental factors on the testosterone levels) – separate analyses for each hour and a combined mixed-model approach.

Individual-hour analyses. These analyses involved:

- fitting the testosterone data for a given hour as a function of the experimental factors, their two-factor interactions, and the baseline level
- examining the significance of the two-factor interactions (2fi)
- choosing (and fitting) a reduced model form by eliminating any 2fi that was not statistically significant in any of the four hourly models.

Results for the original-scale data are summarized in Tables 15 and 16. Table 15 provides an indication of which effects were retained in the reduced model and which of those terms were statistically significant. The lower portion of the table gives statistics characterizing the fit of the models.

Table 15 indicates that mean concentration levels increase with time. The models for the without and with-hCG stimulation data are somewhat different, but both indicate statistical significance for z2 (atmosphere type) and z3 (rat age). For the without-hCG case, the model also shows a significant effect of the covariate and of the z1*z2 (media type by atmosphere type) interaction. For the with-hCG case, the covariate was not statistically significant and there was some (weak) indication of a z1*z3 (media type by rat age) interaction. The RMSE values tend to increase with increasing concentration levels (i.e., with time). The C.V.s, on the other hand, tend to be fairly stable, suggesting that a log-tranform of the concentrations should result in data with approximately homogeneous variances over the various time points.

Adjusted means based on the models that were indicated in Table 15 are presented in Table 16. The means are those that are estimated to occur for a given level of a factor (or given combination of factors) when other effects in the model (e.g., the baseline level covariate) are fixed at their mean values. The three first columns of the table identify the factor levels (see Table 7). Within each set of the levels (e.g., the three rows with z1= -1, 0, and +1), the estimated adjusted mean that is largest is highlighted. Asterisks beside the other non-highlighted means indicate if that particular mean is deemed to be statistically significant from the one that is highlighted. For the non-stimulated data, for instance, the table indicates no significant difference among the z1 levels, although the zero level is consistently estimated to be the largest. For the atmosphere and rat age factors, the zero levels generally have the highest estimated mean testosterone concentrations and the other levels typically have significantly lower means. An exception is the rat age (z3) factor for the hCG-stimulated case, where the 11-week old and the 15-week old rats had similar adjusted mean levels. Even when interactions are considered (lower portion of Table 16), the zero levels of all three factors are either estimated to have the highest adjusted means or to have adjusted means that are not significantly different from the factor combination having the highest estimated mean level.

Tables 17 and 18 show results for the log-transformed data. These tables are analogous to Tables 15 and 16, respectively. The models are somewhat different than those indicated in Table 15. On the log scale, the rat age factor does not appear to be as prominent. Also, the Eagle-MEM (z1=1) medium yields the highest mean levels, although the 199 medium (z1=0) levels are not significantly smaller. The air atmosphere consistently produces lower mean levels. As with the original-scale data, the zero levels of all three factors are either estimated to have the highest adjusted means or to have adjusted means that are not significantly different from the factor combination having the highest estimated mean level.

Mixed-model analyses. For each of two data sets and two types of dependent variables indicated above (see “Data”), these analyses involved several steps. First, we employed a mixed model that included

- the main effects of the experimental factors
- two-factor interactions,
- the baseline testosterone level
- a linear and a quadratic time component
- cross products of the linear and quadratic time components with the main effects
- cross products of the linear and quadratic time components with the 2fi.

For this “full” model, we utilized the SAS PROC MIXED procedure to determine a relevant covariance structure for the data set; in particular, we examined 10 different possible covariance structures, using maximum likelihood estimation, and selected one that appeared to be optimal or near optimal. Using that structure, we estimated fixed effects for all of the above model terms. We then

reduced the model by eliminating non-significant higher-order terms. We then re-examined the covariance structure (again selecting from among 10 possible structures) for this reduced model. Using the selected structure, we estimated the fixed effects in the reduced model (using restricted maximum likelihood estimation) and computed adjusted means for the various factor levels, along with approximate 95% confidence intervals for the means.

The adjusted means for the various cases and factors are given in Tables 19 through 22. These are denoted as EST., where J = 1, 2, 3, or 4 denotes hour. Approximate 95% confidence limits are given in the right portion of the table. The lower and upper limits are denoted as LOWJ and HIJ, respectively, where J = 1, 2, 3, or 4 denotes hour. Unlike the individual-hour analyses, these estimates (and the interval estimates) rely on data from all four hours and also reflect a smoothing over time (due to the assumed quadratic time dependence).

Analyses Directed at Objective 2. Differences in hCG-stimulated and non-stimulated testosterone levels were computed for the 27 trials on an hour-by-hour basis. These differences were then analyzed, by hour, using an initial ANOVA model that included all main effects and 2fi. A reduced ANOVA model was then selected by eliminating those 2fi that were not statistically significant. The original-scale models are summarized in Table 23; no 2fi were deemed necessary, so that the model only includes the main effects. Both atmosphere type and rat age were judged to have impact on the testosterone concentration levels. Table 24 shows the adjusted means derived form the model. The estimated adjusted mean difference that is largest is highlighted. Asterisks beside the other non-highlighted mean differences indicate if that particular difference is deemed to be statistically significant from the one that is highlighted. The media type appears to have little effect, but rat age and atmosphere type are significant factors affecting the with-minus-without-hCG differences.

Tables 25 and 26 show comparable results for the differences of the log-transformed data. In this case, rat age appears less important and atmosphere type is the most dominant factor.

Table 7. Factor Levels in the Phase I Assay Optimization Experiment

Factor Identification	Units	Factor Name	Experimental Levels			Coded Experimental Levels		
			1	2	3	1	2	3
Media Type*		Z1	RPMI-1640	medium-199	Eagles-MEM	-1	0	+1
Atmosphere*		Z2	5% CO_2 / 95% air	5% CO_2 / 95% O_2	air	-1	0	+1
Rat Age*	wks	Z3	11	15	22	-1	0	+1

* Treated as a 3-level discrete factor.

Table 8. Data Listing for Samples Without hCG

Animal	Ear Tag	Set Number	whole testis weight g	testis section g	z1	z2	z 3	y0 = Testos. Conc. Baseline	y1 = Testos. Conc. Hour 1	y2 = Testos. Conc. Hour 2	y3 = Testos. Conc. Hour 3	y4 = Testos. Conc. Hour 4
3	303	A	RIGHT-1.5531	0.2331	-1	-1	-1	0.54	3.23	5.21	6.19	6.11
5	308	B	RIGHT-1.8189	0.2519	-1	-1	0	0.45	4.32	5.02	6.81	8.43
16	317	C	LEFT-2.0203	0.2626	-1	-1	1	0.13	1.17	1.96	2.54	2.94
3	303	D	LEFT-1.5632	0.2688	-1	0	-1	0.43	3.60	5.14	6.48	7.11
5	308	E	LEFT-1.7979	0.2750	-1	0	0	0.36	5.56	7.85	8.30	10.48
19	316	F	RIGHT-2.0218	0.2524	-1	0	1	0.18	2.10	3.77	5.11	6.26
2	304	G	RIGHT-1.7860	0.2692	-1	1	-1	0.58	3.73	4.99	5.72	6.06
7	315	H	RIGHT-1.7847	0.2449	-1	1	0	0.57	5.17	7.01	8.39	9.17
16	317	I	RIGHT-1.9804	0.2603	-1	1	1	0.17	1.70	2.67	3.64	4.79
3	303	J	RIGHT-1.5531	0.2742	0	-1	-1	0.63	5.35	6.27	7.52	8.64
5	308	K	RIGHT-1.8189	0.2349	0	-1	0	0.81	6.60	8.54	10.28	10.97
16	317	L	LEFT-2.0203	0.2502	0	-1	1	0.15	1.10	2.04	2.93	4.15
2	304	M	RIGHT-1.7860	0.2668	0	0	-1	0.86	6.60	9.81	11.35	13.48
5	308	N	LEFT-1.7979	0.2504	0	0	0	0.49	5.58	8.02	10.00	11.23
19	316	O	RIGHT-2.0218	0.2715	0	0	1	0.34	3.50	6.56	8.68	9.65
2	304	P	LEFT-1.7809	0.2577	0	1	-1	0.67	3.11	4.85	5.23	5.58
7	315	Q	RIGHT-1.7847	0.2419	0	1	0	0.78	5.23	6.54	7.29	7.85
16	317	R	RIGHT-1.9804	0.2563	0	1	1	0.13	1.31	1.82	2.46	2.83
3	303	S	LEFT-1.5632	0.2651	1	-1	-1	0.21	3.98	6.10	7.37	10.16
5	308	T	RIGHT-1.8189	0.2371	1	-1	0	0.40	5.33	8.05	9.07	12.25
16	317	U	LEFT-2.0203	0.2586	1	-1	1	0.08	1.01	1.45	1.97	2.33
2	304	V	RIGHT-1.7860	0.2741	1	0	-1	0.48	3.44	5.53	6.98	8.12
5	308	W	LEFT-1.7979	0.2340	1	0	0	0.69	4.91	7.36	10.05	11.72
19	316	X	RIGHT-2.0218	0.2686	1	0	1	0.24	2.31	3.98	5.61	6.55
2	304	Y	LEFT-1.7809	0.2681	1	1	-1	0.45	4.44	5.49	6.71	6.86
7	315	Z	RIGHT-1.7847	0.2560	1	1	0	0.74	5.34	6.15	7.87	8.43
16	317	AA	RIGHT-1.9804	0.2408	1	1	1	0.09	0.84	1.18	1.73	1.74

Table 9. Data Listing for Samples With hCG

Animal	Ear Tag	Set Number	Whole testis weight g	Testis section g	z1	z2	z3	yc0 = Testos. Conc. Baseline	yc1 = Testos. Conc. Hour 1	yc2 = Testos. Conc. Hour 2	yc3 = Testos. Conc. Hour 3	yc4 = Testos. Conc. Hour 4
3	303	AC	RIGHT-1.5531	0.2422	-1	-1	-1	0.54	6.56	16.16	28.30	42.09
5	308	BC	RIGHT-1.8189	0.2690	-1	-1	0	1.11	5.96	9.69	15.19	23.41
16	317	CC	LEFT-2.0203	0.2680	-1	-1	1	0.16	2.03	4.88	8.87	15.93
3	303	DC	LEFT-1.5632	0.2374	-1	0	-1	0.73	5.91	20.96	41.76	51.74
5	308	EC	LEFT-1.7979	0.2398	-1	0	0	0.43	4.39	10.84	22.92	33.45
19	316	FC	RIGHT-2.0218	0.2515	-1	0	1	0.26	3.80	9.94	18.38	24.77
2	304	GC	RIGHT-1.7860	0.2494	-1	1	-1	1.29	4.71	7.14	10.26	13.39
7	315	HC	RIGHT-1.7847	0.2401	-1	1	0	0.62	6.35	8.09	11.90	14.64
16	317	IC	RIGHT-1.9804	0.2642	-1	1	1	0.10	1.30	2.74	3.63	5.13
3	303	JC	RIGHT-1.5531	0.2613	0	-1	-1	0.72	8.26	18.51	31.30	39.20
5	308	KC	RIGHT-1.8189	0.2386	0	-1	0	0.47	7.84	15.75	28.83	43.60
16	317	LC	LEFT-2.0203	0.2672	0	-1	1	0.11	2.19	6.70	11.23	18.38
2	304	MC	RIGHT-1.7860	0.2631	0	0	-1	0.86	9.73	31.45	55.27	77.68
5	308	NC	LEFT-1.7979	0.2461	0	0	0	0.89	7.33	18.38	33.23	59.89
19	316	OC	RIGHT-2.0218	0.2516	0	0	1	0.42	4.15	10.45	18.93	27.32
2	304	PC	LEFT-1.7809	0.2461	0	1	-1	0.61	5.57	9.65	12.75	15.73

(continued)

Table 9. Data Listing for Samples With hCG (continued)

Animal	Ear Tag	Set Number	Whole testis weight g	Testis section g	z1	z2	z3	yc0 = Testos. Conc. Baseline	yc1 = Testos. Conc. Hour 1	yc2 = Testos. Conc. Hour 2	yc3 = Testos. Conc. Hour 3	yc4 = Testos. Conc. Hour 4
7	315	QC	RIGHT-1.7847	0.2618	0	1	0	1.38	7.03	9.69	12.35	13.47
16	317	RC	RIGHT-1.9804	0.2607	0	1	1	0.15	1.03	2.14	2.92	4.01
3	303	SC	LEFT-1.5632	0.2375	1	-1	-1	0.34	6.43	18.23	26.69	38.80
5	308	TC	RIGHT-1.8189	0.2668	1	-1	0	0.65	8.79	18.07	29.25	46.72
16	317	UC	LEFT-2.0203	0.2683	1	-1	1	0.11	1.81	4.44	7.96	12.42
2	304	VC	RIGHT-1.7860	0.2593	1	0	-1	0.66	5.91	15.48	24.92	32.07
5	308	WC	LEFT-1.7979	0.2515	1	0	0	0.90	9.38	25.90	45.21	67.45
19	316	XC	RIGHT-2.0218	0.2595	1	0	1	0.18	3.98	10.13	18.33	27.01
2	304	YC	LEFT-1.7809	0.2591	1	1	-1	0.43	5.17	10.59	16.60	25.20
7	315	ZC	RIGHT-1.7847	0.2684	1	1	0	0.52	7.09	12.48	20.01	27.96
16	317	AAC	RIGHT-1.9804	0.2459	1	1	1	0.12	2.44	5.53	9.16	13.76

Table 10. Summary of Data -- Original Scale, Without hCG

Simple Statistics							
Variable	N	Mean	Std Dev	Sum	Minimum	Maximum	Label
y0	27	0.43148	0.24012	11.65000	0.08000	0.86000	y0 =_Testos._Conc._Baseline
y1	27	3.72444	1.77928	100.56000	0.84000	6.60000	y1 =_Testos._Conc._Hour 1
y2	27	5.30963	2.33770	143.36000	1.18000	9.81000	y2 =_Testos._Conc._Hour 2
y3	27	6.52889	2.67986	176.28000	1.73000	11.35000	y3 =_Testos._Conc._Hour 3
y4	27	7.55148	3.16645	203.89000	1.74000	13.48000	y4 =_Testos._Conc._Hour 4

Pearson Correlation Coefficients, N = 27 Prob > |r| under H0: Rho=0					
	y0	y1	y2	y3	y4
y0 y0 =_Testos._Conc._Baseline	1.00000	0.83359 <.0001	0.77555 <.0001	0.75779 <.0001	0.64894 0.0003
y1 y1 =_Testos._Conc._Hour 1	0.83359 <.0001	1.00000	0.96006 <.0001	0.93735 <.0001	0.89924 <.0001
y2 y2 =_Testos._Conc._Hour 2	0.77555 <.0001	0.96006 <.0001	1.00000	0.98329 <.0001	0.96280 <.0001
y3 y3 =_Testos._Conc._Hour 3	0.75779 <.0001	0.93735 <.0001	0.98329 <.0001	1.00000	0.97499 <.0001
y4 y4 =_Testos._Conc._Hour 4	0.64894 0.0003	0.89924 <.0001	0.96280 <.0001	0.97499 <.0001	1.00000

Table 11. Summary of Data -- Log Scale, Without hCG

Simple Statistics						
Variable	N	Mean	Std Dev	Sum	Minimum	Maximum
ly0	27	-1.04291	0.70964	-28.15853	-2.52573	-0.15082
ly1	27	1.15995	0.62490	31.31878	-0.17435	1.88707
ly2	27	1.53822	0.57738	41.53197	0.16551	2.28340
ly3	27	1.76591	0.52363	47.67970	0.54812	2.42922
ly4	27	1.90916	0.52876	51.54720	0.55389	2.60121

Pearson Correlation Coefficients, N = 27 Prob > |r| under H0: Rho=0					
	ly0	ly1	ly2	ly3	ly4
ly0	1.00000	0.90646 <.0001	0.88113 <.0001	0.86395 <.0001	0.79479 <.0001
ly1	0.90646 <.0001	1.00000	0.97732 <.0001	0.96441 <.0001	0.93255 <.0001
ly2	0.88113 <.0001	0.97732 <.0001	1.00000	0.99193 <.0001	0.97266 <.0001
ly3	0.86395 <.0001	0.96441 <.0001	0.99193 <.0001	1.00000	0.98411 <.0001
ly4	0.79479 <.0001	0.93255 <.0001	0.97266 <.0001	0.98411 <.0001	1.00000

Table 12. Summary of Data -- Original Scale, With hCG

Simple Statistics							
Variable	N	Mean	Std Dev	Sum	Minimum	Maximum	Label
yc0	27	0.54667	0.36060	14.76000	0.10000	1.38000	yc0 =_Testos._Conc._Baseline
yc1	27	5.37556	2.48467	145.14000	1.03000	9.73000	yc1 =_Testos._Conc._Hour 1
yc2	27	12.37074	7.01613	334.01000	2.14000	31.45000	yc2 =_Testos._Conc._Hour 2
yc3	27	20.96852	12.82440	566.15000	2.92000	55.27000	yc3 =_Testos._Conc._Hour 3
yc4	27	30.19333	18.74161	815.22000	4.01000	77.68000	yc4 =_Testos._Conc._Hour 4

Pearson Correlation Coefficients, N = 27 Prob > |r| under H0: Rho=0					
	yc0	yc1	yc2	yc3	yc4
yc0 yc0 =_Testos._Conc._Baseline	1.00000	0.66941 0.0001	0.44159 0.0211	0.38125 0.0497	0.34634 0.0768
yc1 yc1 =_Testos._Conc._Hour 1	0.66941 0.0001	1.00000	0.86213 <.0001	0.80157 <.0001	0.78474 <.0001
yc2 yc2 =_Testos._Conc._Hour 2	0.44159 0.0211	0.86213 <.0001	1.00000	0.98464 <.0001	0.96305 <.0001
yc3 yc3 =_Testos._Conc._Hour 3	0.38125 0.0497	0.80157 <.0001	0.98464 <.0001	1.00000	0.98016 <.0001
yc4 yc4 =_Testos._Conc._Hour 4	0.34634 0.0768	0.78474 <.0001	0.96305 <.0001	0.98016 <.0001	1.00000

Table 13. Summary of Data -- Log Scale, With hCG

Simple Statistics						
Variable	N	Mean	Std Dev	Sum	Minimum	Maximum
lyc0	27	-0.86822	0.80586	-23.44183	-2.30259	0.32208
lyc1	27	1.53650	0.61019	41.48556	0.02956	2.27521
lyc2	27	2.34072	0.64378	63.19938	0.76081	3.44840
lyc3	27	2.83766	0.70489	76.61684	1.07158	4.01223
lyc4	27	3.19518	0.71702	86.26981	1.38879	4.35260

Pearson Correlation Coefficients, N = 27 Prob > |r| under H0: Rho=0					
	lyc0	lyc1	lyc2	lyc3	lyc4
lyc0	1.00000	0.84699 <.0001	0.68581 <.0001	0.61367 0.0007	0.54138 0.0035
lyc1	0.84699 <.0001	1.00000	0.92139 <.0001	0.87024 <.0001	0.82124 <.0001
lyc2	0.68581 <.0001	0.92139 <.0001	1.00000	0.98599 <.0001	0.95954 <.0001
lyc3	0.61367 0.0007	0.87024 <.0001	0.98599 <.0001	1.00000	0.98703 <.0001
lyc4	0.54138 0.0035	0.82124 <.0001	0.95954 <.0001	0.98703 <.0001	1.00000

Table 14. Summary of Statistical Analysis of Baseline Data

		Original-Scale Models		Log-Scale Models	
		Without hCG Stimulation	With hCG Stimulation	Without hCG Stimulation	With hCG Stimulation
Dependent Variable:		Y0	Y0	log(Y0)	log(Y0)
Dependent Variable Mean:		0.431	0.547	-1.043	0.868
Significance of Model Terms:	z1	XX		XXX	
	z2			XX	
	z3	XXX	XXX	XXX	XXX
	z1*z2	na	na	XX	na
	z1*z3	na	na	na	na
	z2*z3	na	na	XX	na
R^2		0.766	0.628	0.950	0.822
RMSE		0.132	0.251	0.233	0.388
C.V.		30.7	45.9	--	--

X = statistically significant effect at 0.10 level of significance.
XX = statistically significant effect at 0.05 level of significance.
XXX = statistically significant effect at 0.01 level of significance.
na = not applicable (effect not included in the model)

Table 15. Summary of ANOCOVA Results for Individual-Hour Original-Scale Models

		Without hCG Stimulation				With hCG Stimulation			
Dependent Variable:		Y1	Y2	Y3	Y4	Y1	Y2	Y3	Y4
Dependent Variable Mean:		3.72	5.31	6.53	7.55	5.38	12.37	20.97	30.19
Significance of Model Terms:	Y0	XX	XX	XX					
	z1					XX	X		
	z2	X	XXX	XXX	XXX	XX	XXX	XXX	XXX
	z3	XXX	XX	XXX	XX	XXX	XXX	XXX	XXX
	z1*z2	XX	XX	XX	X	na	na	na	na
	z1*z3	na	na	na	na	X		X	
R^2		0.931	0.913	0.917	0.877	0.904	0.862	0.865	0.847
RMSE		0.62	0.91	1.02	1.46	1.01	3.43	6.21	9.65
C.V.		16.6	17.1	15.5	19.3	18.9	27.7	29.6	32.0

X = statistically significant effect at 0.10 level of significance.
XX = statistically significant effect at 0.05 level of significance.
XXX = statistically significant effect at 0.01 level of significance.
na = not applicable (effect not included in the model)

Table 16. Adjusted Mean Levels Based on Original-Scale Models

Level of Independent Variable			Mean Levels of Dependent Variables: Without hCG Stimulation				Mean Levels of Dependent Variables: With hCG Stimulation			
z1	z2	z3	Y1 Mean	Y2 Mean	Y3 Mean	Y4 Mean	Y1 Mean	Y2 Mean	Y3 Mean	Y4 Mean
-1			3.55	5.05	6.14	6.99	4.52*	10.08*	18.11	25.30
0			3.95	5.64	6.83	7.92	5.83	13.70	23.41	34.01
+1			3.67	5.24	6.62	7.75	5.77	13.34	21.38	31.26
	-1		3.72	5.16*	6.31**	7.50*	5.62	12.43*	20.40**	30.39**
	0		4.12	6.37	7.97	9.33	6.02	17.10	31.25	45.05
	+1		3.34*	4.40**	5.30**	5.82**	4.49**	7.59**	11.25**	15.14**
		-1	3.86**	5.53*	6.59*	7.67*	6.34	16.58	28.33	38.71
		0	4.89	6.58	7.99	9.56	6.91	14.50	25.61	38.99
		+1	2.43**	3.83**	5.01**	5.43**	2.88**	6.03**	8.97**	12.88**
-1	-1		3.07**	4.28**	5.44**	6.01**				
-1	0		4.06	6.00	7.11*	8.30				
-1	+1		3.51*	4.86**	5.88**	6.65**				
0	-1		4.07	5.24**	6.48**	7.60*				
0	0		4.85	7.63	9.43	11.03				
0	+1		2.94**	4.04**	4.57**	5.11**				
+1	-1		4.02	5.96	7.02*	8.89				
+1	0		3.44*	5.48*	7.38*	8.67				
+1	+1		3.55*	4.29**	5.46**	5.69**				
-1		-1					5.43**	15.00	28.51	38.78
-1		0					5.40**	9.68**	17.65**	25.55**
-1		+1					2.73**	5.55**	8.18**	11.58**
0		-1					7.68	20.02	34.14	46.02
0		0					7.05	14.90	26.88	42.62
0		+1					2.76**	6.17**	9.21**	13.40**
+1		-1					5.90*	14.71	22.34*	31.33*
+1		0					8.28	18.93	32.30	48.80
+1		+1					3.13**	6.37**	9.50**	13.67**

Shaded cell indicates highest mean estimated level.

* Indicates that the mean level is significantly lower than the cell with the maximum estimated level, p=0.05.

** Indicates that the mean level is significantly lower than the cell with the maximum estimated level, p=0.01.

Table 17. Summary of ANOCOVA Results for Individual-Hour Log-Scale Models

		Without hCG Stimulation				With hCG Stimulation			
Dependent Variable:		log(Y1)	log(Y2)	log(Y3)	log(Y4)	log(Y1)	log(Y2)	log(Y3)	log(Y4)
Dependent Variable Mean:		1.160	1.538	1.766	1.909	1.537	2.341	2.838	3.195
Significance of Model Terms:	log(Y0)	XXX	XXX	XXX	XXX	XX			
	z1					X	XX	X	X
	z2	X	XXX	XXX	XXX	XX	XXX	XXX	XXX
	z3						X	X	X
	z1*z2	XX	XX	XX	XX			X	X
	z1*z3		X	X	XX	na	na	na	na
R^2		0.963	0.964	0.962	0.951	0.894	0.900	0.917	0.924
RMSE		0.185	0.167	0.156	0.181	0.261	0.268	0.268	0.260

X = statistically significant effect at 0.10 level of significance.
XX = statistically significant effect at 0.05 level of significance.
XXX = statistically significant effect at 0.01 level of significance.
na = not applicable (effect not included in the model)

Table 18. Adjusted Mean Levels Based on Log-Scale Models

Level of Independent Variable			Mean Levels of Dependent Variables: Without hCG Stimulation				Mean Levels of Dependent Variables: With hCG Stimulation			
z1	z2	z3	log(Y1) Mean	log(Y2) Mean	log(Y3) Mean	log(Y4) Mean	log(Y1) Mean	log(Y2) Mean	log(Y3) Mean	log(Y4) Mean
-1			1.162	1.551	1.764	1.903	1.381*	2.147*	2.669*	3.021*
0			1.085	1.456	1.679	1.834	1.527	2.362	2.836	3.190
+1			1.232	1.608	1.855	1.990	1.702	2.513	3.008	3.374
	-1		1.208	1.591	1.810	1.98	1.628	2.431	2.949*	3.356*
	0		1.240	1.690	1.935	2.10	1.644	2.696	3.305	3.687
	+1		1.031*	1.334**	1.552**	1.64**	1.337*	1.895**	2.259**	2.542**
		-1	1.096	1.442	1.640	1.780	1.651	2.610	3.098	3.444
		0	1.279	1.550	1.774	1.956	1.698	2.463	2.982	3.408
		+1	1.105	1.623	1.884	1.991	1.261	1.949*	2.433*	2.734*
-1	-1		1.021*	1.406**	1.645**	1.752**	1.419	2.192*	2.730*	3.211*
-1	0		1.371	1.805	1.993	2.157	1.515	2.566	3.251	3.552
-1	+1		1.094*	1.440*	1.654*	1.801*	1.208*	1.682**	2.026**	2.302**
0	-1		1.066*	1.403*	1.659*	1.857*	1.755	2.582	3.124	3.477
0	0		1.292	1.740	1.981	2.144	1.675	2.782	3.379	3.864
0	+1		0.898**	1.225**	1.396**	1.501**	1.151*	1.723**	2.005**	2.229**
+1	-1		1.539	1.963	2.127	2.341	1.711	2.520	2.991	3.381
+1	0		1.057*	1.524*	1.833	2.001	1.743	2.740	3.285	3.645
+1	+1		1.102*	1.338**	1.606**	1.628**	1.651	2.281	2.747*	3.097**
-1		-1	0.946*	1.307*	1.512*	1.587**				
-1		0	1.401	1.658	1.855	2.053				
-1		+1	1.139	1.686	1.925	2.070				
0		-1	0.982*	1.288*	1.470*	1.650*				
0		0	1.213	1.470	1.685	1.821				
0		+1	1.061	1.609	1.882	2.031				
+1		-1	1.359	1.730	1.939	2.104				
+1		0	1.221	1.521	1.781	1.996				
+1		+1	1.117	1.572	1.846	1.871				

Shaded cell indicates highest mean estimated level.

* Indicates that the mean level is significantly lower than the cell with the maximum estimated level, p=0.05.
** Indicates that the mean level is significantly lower than the cell with the maximum estimated level, p=0.01.

Table 19. Least Squares Means for Reduced Log-scale Model: Without hCG
Mean covariate value: ly0=-1.043

x1	x2	x3	EST1	EST2	EST3	EST4	LOW 1	HI1	LOW 2	HI2	LOW 3	HI3	LOW 4	HI4
-1	_	_	1.163	1.527	1.773	1.901	1.059	1.267	1.424	1.629	1.670	1.875	1.797	2.005
0	_	_	1.116	1.471	1.708	1.828	0.942	1.290	1.298	1.644	1.536	1.881	1.655	2.002
1	_	_	1.211	1.587	1.846	1.988	1.064	1.357	1.442	1.733	1.701	1.992	1.842	2.134
_	-1	_	1.184	1.563	1.824	1.968	1.039	1.330	1.418	1.708	1.680	1.969	1.823	2.114
_	0	_	1.278	1.672	1.949	2.109	1.146	1.409	1.542	1.802	1.819	2.080	1.978	2.240
_	1	_	1.028	1.350	1.554	1.640	0.928	1.128	1.251	1.448	1.455	1.652	1.540	1.741
_	_	-1	1.137	1.472	1.683	1.771	0.901	1.374	1.236	1.708	1.448	1.919	1.534	2.007
_	_	0	1.327	1.606	1.813	1.949	1.031	1.622	1.311	1.901	1.518	2.108	1.654	2.245
_	_	1	1.026	1.507	1.831	1.998	0.523	1.529	1.004	2.010	1.328	2.334	1.494	2.501

Reduced model was fit using compound symmetry covariance structure.
Effects retained in the reduced model were the following (tL denote the linear time effect, tQ denotes the quadratic):
ly0, z1, z2, z3, z1*z2, z1*z3, tL, tL*z1, tL*z2, tL*z3, tL*z1*z3, tQ, tQ*z3.

Table 20. Least Squares Means for Reduced Original-scale Model: Without hCG
Mean covariate value: y0=0.43

x1	x2	x3	EST 1	EST 2	EST 3	EST 4	LO W1	HI1	LO W2	HI2	LO W3	HI3	LO W4	HI4
-1	_	_	3.70	5.11	6.24	7.09	2.99	4.40	4.41	5.80	5.55	6.94	6.39	7.79
0	_	_	3.74	5.35	6.67	7.72	2.82	4.66	4.43	6.27	5.75	7.59	6.79	8.64
1	_	_	3.76	5.40	6.75	7.82	3.05	4.48	4.69	6.10	6.04	7.46	7.11	8.53
_	-1	_	3.78	5.30	6.54	7.50	3.07	4.48	4.60	6.00	5.84	7.24	6.80	8.21
_	0	_	4.17	6.18	7.91	9.36	3.54	4.81	5.56	6.81	7.29	8.54	8.73	9.99
_	1	_	3.25	4.37	5.21	5.77	2.59	3.90	3.72	5.01	4.56	5.85	5.12	6.42
_	_	-1	3.65	5.20	6.47	7.46	2.74	4.57	4.29	6.12	5.56	7.38	6.54	8.38
_	_	0	4.45	6.30	7.87	9.15	3.29	5.62	5.14	7.46	6.70	9.03	7.99	10.32
_	_	1	3.09	4.35	5.32	6.02	1.31	4.87	2.57	6.13	3.55	7.10	4.24	7.80

Reduced model was fit using compound symmetry covariance structure.
Effects retained in the reduced model were the following (tL denote the linear time effect, tQ denotes the quadratic):
y0, z1, z2, z3, z1*z2, z1*z3, z2*z3, tL, tL*z1, tL*z2, tL*z3, tL*z1*z2, tL*z1*z3, tL*z2*z3, tQ.

Table 21. Least Squares Means for Reduced Log-scale Model: with hCG
Mean covariate value: lyc0=-0.868

x1	x2	x3	EST1	EST2	EST3	EST4	LOW 1	HI1	LOW 2	HI2	LOW 3	HI3	LOW 4	HI4
-1	_	_	1.388	2.153	2.694	3.012	1.199	1.576	1.970	2.335	2.512	2.876	2.823	3.200
0	_	_	1.554	2.314	2.852	3.166	1.360	1.748	2.126	2.503	2.664	3.040	2.972	3.360
1	_	_	1.668	2.471	3.051	3.408	1.469	1.867	2.278	2.665	2.858	3.244	3.209	3.607
_	-1	_	1.598	2.400	2.996	3.385	1.402	1.795	2.206	2.595	2.801	3.190	3.188	3.581
_	0	_	1.682	2.662	3.319	3.651	1.481	1.884	2.463	2.862	3.119	3.518	3.449	3.852
_	1	_	1.329	1.876	2.283	2.550	1.141	1.518	1.690	2.062	2.097	2.469	2.361	2.738
_	_	-1	1.726	2.560	3.109	3.372	1.489	1.963	2.325	2.795	2.873	3.344	3.135	3.609
_	_	0	1.793	2.443	2.951	3.317	1.530	2.056	2.182	2.704	2.690	3.212	3.054	3.580
_	_	1	1.091	1.935	2.537	2.896	0.713	1.470	1.558	2.313	2.159	2.914	2.518	3.275

Reduced model was fit using first-order autoregressive covariance structure.
Effects retained in the reduced model were the following (tL denote the linear time effect, tQ denotes the quadratic):
lyc0, z1, z2, z3, z1*z2, tL, tL*z1, tL*z2, tL*z3, tL*z1*z2, tQ, tQ*z2, tQ*z3.

Table 22. Least Squares Means for Reduced Original-scale Model: with hCG
Mean covariate value: yc0=0.55

x1	x2	x3	EST 1	EST2	EST3	EST4	LO W1	HI1	LOW 2	HI2	LOW 3	HI3	LOW 4	HI4
-1	_	_	5.11	12.20	20.55	30.17	4.43	5.79	10.49	13.90	17.76	23.34	26.03	34.31
0	_	_	5.52	12.60	20.96	30.57	4.83	6.21	10.90	14.31	18.17	23.75	26.43	34.71
1	_	_	5.59	12.68	21.03	30.65	4.89	6.29	10.97	14.39	18.24	23.82	26.51	34.79
_	-1	_	5.68	12.69	20.97	30.52	4.71	6.64	9.80	15.59	16.09	25.85	23.51	37.52
_	0	_	6.14	17.64	30.40	44.43	5.18	7.09	14.75	20.53	25.52	35.28	37.43	51.44
_	1	_	4.40	7.15	11.16	16.44	3.45	5.36	4.26	10.04	6.28	16.04	9.43	23.45
_	_	-1	6.32	16.54	27.02	37.76	5.36	7.29	13.66	19.42	22.24	31.81	30.62	44.90
_	_	0	6.78	14.01	23.91	36.48	5.79	7.78	11.12	16.90	19.12	28.70	29.34	43.63
_	_	1	3.11	6.92	11.60	17.15	2.04	4.17	4.01	9.84	6.80	16.41	9.99	24.30

Reduced model was fit using first-order autoregressive covariance structure with heterogeneous variances.
Effects retained in the reduced model were the following (tL denote the linear time effect, tQ denotes the quadratic):
y0, z1, z2, z3, tL, tL*z2, tL*z3, tQ, tQ*z3.

Table 23. Analysis of Differences in Levels for With and Without hCG Stimulation: Original-Scale Models

Dependent Variable:		diff(Y0)	diff(Y1)	diff(Y2)	diff(Y3)	diff(Y4)
Dependent Variable Mean:		0.12	1.65	7.06	14.44	22.64
Significance of Model Terms:	z1					
	z2			XXX	XXX	XXX
	z3		XX	XXX	XXX	XXX
R^2		0.175	0.457	0.687	0.722	0.712
RMSE		0.25	1.05	3,49	6,57	10.04

X = statistically significant effect at 0.10 level of significance.
XX = statistically significant effect at 0.05 level of significance.
XXX = statistically significant effect at 0.01 level of significance.

Table 24. Adjusted Mean Differences (With-Without hCG), Based on Original-Scale Models

Level of Independent Variable			Mean Levels of Dependent Variables				
z1	z2	z3	Mean diff (Y0)	Mean diff (Y1)	Mean diff (Y2)	Mean diff (Y3)	Mean diff (Y4)
-1			0.20	1.16	5.20	12.00	18.13
0			0.08	1.64	7.59	15.67	24.99
+1			0.06	2.16	8.40	15.64	24.80
	-1		0.09	1.98	7.53	14.77*	23.84*
	0		0.14	1.89	10.61	22.93	35.20
	+1		0.12	1.09	3.04**	5.62**	8.89**
		-1	0.15	2.31	10.53	20.48	29.31
		0	0.19	1.79	7.15	15.65	26.67
		+1	0.01	0.85**	3.50**	7.19**	11.94**

Shaded cell indicates highest mean estimated level.
* Indicates that the mean level is significantly lower than the cell with the maximum estimated level, p=0.05.
** Indicates that the mean level is significantly lower than the cell with the maximum estimated level, p=0.01.

Table 25. Analysis of Differences in Levels for With and Without hCG Stimulation: Log-Scale Models

Dependent Variable:		diff (log(Y0))	diff (log(Y1))	diff (log(Y2))	diff (log(Y3))	diff (log(Y4))
Dependent Variable Mean:		0.175	0.377	0.802	1.072	1.286
Significance of Model Terms:	z1			X		
	z2			XX	XXX	XXX
	z3					
R^2		0.142	0.305	0.528	0.585	0.503
RMSE		0.393	0.289	0.313	0.335	0.405

X = statistically significant effect at 0.10 level of significance.
XX = statistically significant effect at 0.05 level of significance.
XXX = statistically significant effect at 0.01 level of significance.

Table 26. Adjusted Mean Differences (With-Without hCG), Based on Log-Scale Models

Level of Independent Variable			Mean Levels of Dependent Variables				
z1	z2	z3	Mean diff log(Y0)	Mean diff log(Y1)	Mean diff log(Y2)	Mean diff log(Y3)	Mean diff log(Y4)
-1			0.282	0.289	0.659**	0.963	1.166
0			0.079	0.307	0.732	0.995	1.197
+1			0.164	0.533	1.017	1.278	1.496
	-1		0.186	0.493	0.956	1.248	1.486
	0		0.242	0.380	0.931	1.298	1.503
	+1		0.096	0.257	0.520**	0.670**	0.869**
		-1	0.236	0.446	0.957	1.259	1.447
		0	0.243	0.274	0.637*	0.948	1.170
		+1	0.045	0.410	0.814	1.008	1.241

Shaded cell indicates highest mean estimated level.
* Indicates that the mean level is significantly lower than the cell with the maximum estimated level, p=0.05.
** Indicates that the mean level is significantly lower than the cell with the maximum estimated level, p=0.01.

Statistical Analysis of the Phase I B Assay Optimization Experiment

Objectives

Since the gaseous atmosphere of 5% CO_2 / 95% O_2 was optimal and it was thought that most laboratories would not have incubators to accommodate this mixture, a comparison was made between incubated samples and those in media that had be gassed with the mixture.

The Phase IB assay optimization experiment involved assessing the effect of a single experimental factor – using gassed or incubated samples. All other factors were held fixed. Ten trials of each condition were run both with and without hCG stimulation. For each trial, repeated measurements are taken at baseline (time 0) and at 1, 2, 3, and 4 hours after baseline. Objective of the experiment was to assess whether the treatments differed in terms of the resultant testosterone levels.

Data

Two basic SAS data sets were constructed from the raw data and two fundamental types of dependent variables were used in the analyses of each type:

Date Set 1: Cases without hCG stimulation

Dependent variables: testosterone concentrations

Dependent variables: (natural) logarithm of testosterone concentrations

Data Set 2: Cases with hCG stimulation

Dependent variables: testosterone concentrations

Dependent variables: (natural) logarithm of testosterone concentrations

Each data set can be viewed as consisting of 20 observations (rows). Each observation includes dependent variable values for 4 time points and a corresponding baseline level. Each observation also includes data identifying the levels of the pertinent factors. Data are listed in Table 27 for the unchallenged samples, and in Table 28, for the challenged cases.

Statistical Analysis Methods

Analysis of variance (ANOVA) were used to analyze the data for each individual time point (including the baseline). ANOVAs were performed for both original-scale data and log-scaled data (natural logarithm). Analysis of covariance (ANOCOVA) models utilizing the baseline level (or log-level) as a covariate were also employed.

Results

Overall Characterization of the Data. Table 29 provides summary statistics characterizing the testosterone levels in the non-hCG-stimulated data set. This summary ignores the experimental factor. The top portion of the table gives, by hour, the sample size (n), the mean, standard deviation, sum, minimum, and maximum. These variables are denoted as yJ, where J denotes the hour and takes on values of 0, 1, 2, 3, and 4. The lower portion of the table gives the correlations between the hourly data. The following trends are apparent:

- the means continue to increase over time
- the standard deviations also increase over time (i.e., as the mean level gets larger)
- the correlations are generally high, and tend to be largest for adjacent hours.

Table 30 provides a similar summary for the log-scaled data; these variables are denoted as lyJ, where J denotes the hour. Similar trends for the means and correlations are evident, but the standard deviations tend to be fairly stable across the various time points.

Tables 31 and 32 furnish comparable information for the hCG-stimulated samples. Similar trends are evident for these data. Mean levels tend to be much higher than for the non-stimulated samples.

Analysis of Variance and Covariance. Means, by hour and sample condition, are presented in Table 33 for the non-hCG-stimulated original-scale data. The table gives the number of observations, the approximate 95% confidence limits for the mean, the estimated mean and standard deviation. The last column gives the mean (for hours 1, 2, 3, and 4) adjusted for the baseline level, as determined from the ANOCOVA. Tables 34, 35, and 36 give corresponding results for the log-scaled data and the hCG-stimulated cases. The table below summarizes the findings detailed in Tables 33 through 36. For the most part, the ANOVAs and ANOCOVAs of the hourly data did not detect significant differences between the testosterone levels of the gassed and incubated samples. If the 0.05 significance level is used to judge statistical significance, then only the baseline case of Table 33 yielded a significant difference.

Source	**Type Data Analyzed**	**ANOVA Results**	**ANOCOVA Results**
Table 33	Original scale, non-hCG-stimulated	Baseline gassed samples have lower testosterone mean (p=0.05); all other hours not significantly different at 0.10 level.	Hour-1 and hour-4 gassed samples have higher testosterone means (p=0.08 and 0.07, respectively); other hours not significantly different at 0.10 level.
Table 34	Log scale, non-hCG-stimulated	Baseline gassed samples have lower testosterone mean (p=0.06); all other hours not significantly different at 0.10 level.	No significant differences at 0.10 level.
Table 35	Original scale, hCG-stimulated	No significant differences at 0.10 level.	No significant differences at 0.10 level.
Table 36	Log scale, hCG-stimulated	No significant differences at 0.10 level.	No significant differences at 0.10 level.

Table 27. Phase IB Data: Samples Without hCG

Ear Tag	Body Weight g	Testis wt g	Section wt g	Run Number	x0	y0 =Testos. Conc. Baseline	y1 =Testos. Conc. Hour 1	y2 =Testos. Conc. Hour 2	y3 =Testos. Conc. Hour 3	y4 =Testos. Conc. Hour 4
320	361.74	RIGHT - 1.5641	0.2413	1	1	0.35	3.49	5.46	6.66	8.05
321	361.74	LEFT - 1.5303	0.2657	2	1	0.38	3.92	4.95	6.88	8.27
321	365.84	RIGHT - 1.8176	0.2623	3	1	0.36	4.66	6.52	7.88	8.53
321	365.84	LEFT - 1.8273	0.2463	4	1	0.34	3.94	4.94	6.30	7.71
321	365.84	LEFT - 1.8273	0.2389	5	1	0.32	4.53	6.36	7.56	7.73
322	372.13	RIGHT - 1.5835	0.2341	6	1	0.14	3.30	4.34	5.65	6.40
322	372.13	LEFT - 1.5263	0.2436	7	1	0.18	1.67	2.47	3.16	3.63
323	365.46	RIGHT - 1.7040	0.2722	8	1	0.45	3.87	5.41	6.06	7.90
324	356.94	RIGHT - 1.4908	0.2626	9	1	0.42	5.32	7.49	9.71	11.01
323	365.46	LEFT - 1.6760	0.2711	10	1	0.35	5.32	7.07	8.83	10.17
320	361.74	RIGHT - 1.5641	0.2713	11	2	0.49	5.06	7.37	9.13	10.97
320	361.74	LEFT - 1.5303	0.2543	12	2	0.38	2.94	4.36	5.44	6.45
321	365.84	RIGHT - 1.8176	0.2610	13	2	0.38	4.76	6.78	8.51	9.21
321	365.84	LEFT - 1.8273	0.2408	14	2	0.36	3.64	5.37	6.34	6.87
322	372.13	RIGHT - 1.5835	0.2472	15	2	0.33	3.38	4.87	5.53	6.41
322	372.13	LEFT - 1.5263	0.2499	16	2	0.46	4.45	5.56	6.85	8.15
323	365.46	RIGHT - 1.7040	0.2474	17	2	0.40	4.26	6.30	7.99	9.13
323	365.46	LEFT - 1.6760	0.2630	18	2	0.39	3.56	5.63	6.78	7.71
324	356.94	RIGHT - 1.4908	0.2365	19	2	0.37	2.62	3.69	4.82	5.84
324	356.94	LEFT - 1.4136	0.2684	20	2	0.49	4.56	5.85	6.55	9.02

x0 = sample condition: 1 = gassed, 2 = incubated

Table 28. Phase IB Data: Samples With hCG

Ear Tag	Body Weight g	Testis wt g	Section wt g	Run Number	x0	y0 =Testos. Conc. Baseline	y1 =Testos. Conc. Hour 1	y2 =Testos. Conc. Hour 2	y3 =Testos. Conc. Hour 3	y4 =Testos. Conc. Hour 4
320	361.74	RIGHT - 1.5641	0.2387	1	1	0.36	9.56	20.60	32.84	41.13
320	361.74	LEFT - 1.5303	0.2727	2	1	0.52	6.31	13.49	22.25	29.13
321	365.84	RIGHT - 1.8176	0.2518	3	1	0.40	5.12	13.29	18.32	25.33
321	365.84	LEFT - 1.8273	0.2748	4	1	0.48	7.57	14.92	23.79	30.83
321	365.84	LEFT - 1.8273	0.2681	5	1	0.43	4.84	9.66	13.47	18.59
322	372.13	RIGHT - 1.5835	0.2429	6	1	0.28	8.73	18.74	30.71	45.84
322	372.13	LEFT - 1.5263	0.2523	7	1	0.23	4.18	9.38	12.68	18.38
322	365.46	RIGHT - 1.7040	0.2550	8	1	0.42	5.95	12.41	21.00	30.28
324	356.94	RIGHT - 1.4908	0.2489	9	1	0.12	5.09	11.99	17.25	28.43
323	365.46	LEFT - 1.6760	0.2513	10	1	0.21	3.40	8.04	13.78	19.14
320	361.74	RIGHT - 1.5641	0.2451	11	2	0.34	6.71	17.75	29.37	39.76
320	361.74	LEFT - 1.5303	0.2409	12	2	0.38	5.07	12.01	17.63	23.46
321	365.84	RIGHT - 1.8176	0.2471	13	2	0.24	7.90	17.49	27.99	39.46
321	365.84	LEFT - 1.8273	0.2695	14	2	0.41	7.52	19.79	30.29	43.69
322	372.13	RIGHT - 1.5835	0.2452	15	2	0.37	4.24	10.43	14.70	19.41
322	372.13	LEFT - 1.5263	0.2635	16	2	0.31	5.06	11.70	19.98	29.90
323	365.46	RIGHT - 1.7040	0.2615	17	2	0.44	5.71	13.87	21.35	30.16
323	365.46	LEFT - 1.6760	0.2328	18	2	0.72	6.93	21.00	31.05	40.41
324	356.94	RIGHT - 1.4908	0.2632	19	2	0.81	5.73	13.45	23.01	34.28
324	356.94	LEFT - 1.4136	0.2491	20	2	0.31	6.14	16.68	26.65	33.43

x0 = sample condition: 1 = gassed, 2 = incubated

Table 29. Summary of Data -- Original Scale, Without hCG

Simple Statistics							
Variable	N	Mean	Std Dev	Sum	Minimum	Maximum	Label
Y0	20	0.36700	0.08652	7.34000	0.14000	0.49000	y0 =_Testos._Conc._Baseline
Y1	20	3.96250	0.92905	79.25000	1.67000	5.32000	y1 =_Testos._Conc._Hour 1
Y2	20	5.53950	1.26145	110.79000	2.47000	7.49000	y2 =_Testos._Conc._Hour 2
Y3	20	6.83150	1.57510	136.63000	3.16000	9.71000	y3 =_Testos._Conc._Hour 3
Y4	20	7.95800	1.76957	159.16000	3.63000	11.01000	y4 =_Testos._Conc._Hour 4

Pearson Correlation Coefficients, N = 20Prob > |r| under H0: Rho=0					
	Y0	Y1	Y2	Y3	Y4
Y0y0 =_Testos._Conc._Baseline	1.00000	0.56839 0.0089	0.57435 0.0081	0.51050 0.0215	0.65502 0.0017
Y1y1 =_Testos._Conc._Hour 1	0.56839 0.0089	1.00000	0.95710 <.0001	0.94357 <.0001	0.94257 <.0001
Y2y2 =_Testos._Conc._Hour 2	0.57435 0.0081	0.95710 <.0001	1.00000	0.97439 <.0001	0.94352 <.0001
Y3y3 =_Testos._Conc._Hour 3	0.51050 0.0215	0.94357 <.0001	0.97439 <.0001	1.00000	0.95462 <.0001
Y4y4 =_Testos._Conc._Hour 4	0.65502 0.0017	0.94257 <.0001	0.94352 <.0001	0.95462 <.0001	1.00000

Table 30. Summary of Data -- Log Scale, Without hCG

Simple Statistics						
Variable	N	Mean	Std Dev	Sum	Minimum	Maximum
ly0	20	-1.03875	0.30294	-20.77491	-1.96611	-0.71335
ly1	20	1.34511	0.27439	26.90217	0.51282	1.67147
ly2	20	1.68265	0.26153	33.65299	0.90422	2.01357
ly3	20	1.89300	0.25530	37.86004	1.15057	2.27316
ly4	20	2.04705	0.25043	40.94100	1.28923	2.39880

Pearson Correlation Coefficients, N = 20Prob > |r| under H0: Rho=0					
	ly0	ly1	ly2	ly3	ly4
ly0	1.00000	0.58422 0.0068	0.60978 0.0043	0.55761 0.0106	0.659980.0015
ly1	0.58422 0.0068	1.00000	0.96741 <.0001	0.95797 <.0001	0.95831<.0001
ly2	0.60978 0.0043	0.96741 <.0001	1.00000	0.97796 <.0001	0.95576<.0001
ly3	0.55761 0.0106	0.95797 <.0001	0.97796 <.0001	1.00000	0.96540<.0001
ly4	0.65998 0.0015	0.95831 <.0001	0.95576 <.0001	0.96540 <.0001	1.00000

Table 31. Summary of Data -- Original Scale, With hCG

Simple Statistics							
Variable	N	Mean	Std Dev	Sum	Minimum	Maximum	Label
YC0	20	0.38900	0.16189	7.78000	0.12000	0.81000	yc0 =_Testos._Conc._Baseline
YC1	20	6.08800	1.58725	121.76000	3.40000	9.56000	yc1 =_Testos._Conc._Hour 1
YC2	20	14.33450	3.87932	286.69000	8.04000	21.00000	yc2 =_Testos._Conc._Hour 2
YC3	20	22.40550	6.48074	448.11000	12.68000	32.84000	yc3 =_Testos._Conc._Hour 3
YC4	20	31.05200	8.66685	621.04000	18.38000	45.84000	yc4 =_Testos._Conc._Hour 4

Pearson Correlation Coefficients, N = 20Prob > |r| under H0: Rho=0					
	YC0	YC1	YC2	YC3	YC4
YC0yc0 =_Testos._Conc._Baseline	1.00000	0.14785 0.5339	0.24875 0.2903	0.25182 0.2841	0.19761 0.4037
YC1yc1 =_Testos._Conc._Hour 1	0.14785 0.5339	1.00000	0.88953 <.0001	0.90763 <.0001	0.87874 <.0001
YC2yc2 =_Testos._Conc._Hour 2	0.24875 0.2903	0.88953 <.0001	1.00000	0.97501 <.0001	0.93343 <.0001
YC3yc3 =_Testos._Conc._Hour 3	0.25182 0.2841	0.90763 <.0001	0.97501 <.0001	1.00000	0.96812 <.0001
YC4yc4 =_Testos._Conc._Hour 4	0.19761 0.4037	0.87874 <.0001	0.93343 <.0001	0.96812 <.0001	1.00000

Table 32. Summary of Data – Log Scale, With hCG

Simple Statistics						
Variable	N	Mean	Std Dev	Sum	Minimum	Maximum
lyc0	20	-1.02614	0.42653	-20.52279	-2.12026	-0.21072
lyc1	20	1.77407	0.26184	35.48142	1.22378	2.25759
lyc2	20	2.62709	0.27611	52.54184	2.08443	3.04452
lyc3	20	3.06710	0.30327	61.34207	2.54003	3.49165
lyc4	20	3.39608	0.29444	67.92156	2.91126	3.82516

Pearson Correlation Coefficients, N = 20Prob > |r| under H0: Rho=0					
	lyc0	lyc1	lyc2	lyc3	lyc4
lyc0	1.00000	0.25901 0.2702	0.29545 0.2060	0.30624 0.1891	0.211980. 3696
lyc1	0.25901 0.2702	1.00000	0.91838 <.0001	0.91443 <.0001	0.89007<. 0001
lyc2	0.29545 0.2060	0.91838 <.0001	1.00000	0.96817 <.0001	0.93546<. 0001
lyc3	0.30624 0.1891	0.91443 <.0001	0.96817 <.0001	1.00000	0.97426<. 0001
lyc4	0.21198 0.3696	0.89007 <.0001	0.93546 <.0001	0.97426 <.0001	1.00000

Table 33. Summary of Results by Sample Condition -- Original Scale, Without hCG

x0	N Obs	Variable	Lower 95% CL for Mean	Upper 95% CL for Mean	Mean	Std Dev	ANOCOVA Adjusted Mean
1	10	Y0	0.26	0.40	**0. 33**	0.10	
		Y1	3.23	4.77	4.00	1.08	**4.30**
		Y2	4.45	6.55	5.50	1.47	5.89
		Y3	5.57	8.17	6.87	1.82	7.32
		Y4	6.51	9.37	7.94	2.00	**8.57**
2	10	Y0	0.37	0.44	**0.41**	0.06	
		Y1	3.34	4.51	3.92	0.81	**3.62**
		Y2	4.79	6.36	5.58	1.10	5.19
		Y3	5.80	7.79	6.79	1.39	6.34
		Y4	6.82	9.13	7.98	1.62	**7.34**

x0 = sample condition: 1 = gassed, 2 = incubated
Bolded entries are statistically significant at the 0.10 level.
Bolded and underlined entries are statistically significant at the 0.05 level.

Table 34. Summary of Results by Sample Condition -- Log Scale, Without hCG

x0	N Obs	Variable	Lower 95% CL for Mean	Upper 95% CL for Mean	Mean	Std Dev	ANOCOVA Adjusted Mean
1	10	ly0	-1.433	-0.898	**-1.165**	0.374	
		ly1	1.105	1.583	1.344	0.334	1.426
		ly2	1.437	1.893	1.665	0.318	1.743
		ly3	1.667	2.110	1.889	0.309	1.961
		ly4	1.820	2.252	2.036	0.302	2.118
2	10	ly0	-1.008	-0.816	**-0.912**	0.134	
		ly1	1.190	1.502	1.346	0.218	1.264
		ly2	1.553	1.848	1.700	0.206	1.623
		ly3	1.751	2.044	1.897	0.205	1.825
		ly4	1.914	2.202	2.058	0.202	1.976

x0 = sample condition: 1 = gassed, 2 = incubated
Bolded entries are statistically significant at the 0.10 level.
Bolded and underlined entries are statistically significant at the 0.05 level.

Table 35. Summary of Results by Sample Condition -- Original Scale, With hCG

x0	N Obs	Variable	Lower 95% CL for Mean	Upper 95% CL for Mean	Mean	Std Dev	ANOCOVA Adjusted Mean
1	10	YC0	0.25	0.44	0.35	0.13	
		YC1	4.65	7.50	6.08	1.99	6.14
		YC2	10.39	16.12	13.25	4.00	13.44
		YC3	15.60	25.62	20.61	7.00	20.94
		YC4	22.10	35.31	28.71	9.23	29.02
2	10	YC0	0.30	0.57	0.43	0.18	
		YC1	5.27	6.93	6.10	1.16	6.03
		YC2	12.82	18.01	15.42	3.62	15.22
		YC3	20.13	28.28	24.20	5.69	23.87
		YC4	27.80	38.99	33.40	7.82	33.09

x0 = sample condition: 1 = gassed, 2 = incubated
Bolded entries are statistically significant at the 0.10 level.
Bolded and underlined entries are statistically significant at the 0.05 level.

Table 36. Summary of Results by Sample Condition – Log Scale, With hCG

x0	N Obs	Variable	Lower 95% CL for Mean	Upper 95% CL for Mean	Mean	Std Dev	ANOCOVA Adjusted Mean
1	10	lyc0	-1.474	-0.818	-1.146	0.458	
		lyc1	1.524	1.989	1.757	0.325	1.776
		lyc2	2.331	2.757	2.544	0.297	2.562
		lyc3	2.737	3.214	2.975	0.334	2.995
		lyc4	3.085	3.538	3.312	0.317	3.323
2	10	lyc0	-1.176	-0.637	-0.906	0.377	
		lyc1	1.652	1.931	1.792	0.195	1.772
		lyc2	2.539	2.881	2.710	0.239	2.693
		lyc3	2.978	3.340	3.159	0.253	3.139
		lyc4	3.295	3.665	3.480	0.259	3.469

x0 = sample condition: 1 = gassed, 2 = incubated
Bolded entries are statistically significant at the 0.10 level.
Bolded and underlined entries are statistically significant at the 0.05 level.

6.0 DISCUSSION

The optimization of the sliced testis assay is necessary in order to proceed to the pre-validation and validation stages of the testing of the assay for use in the Tier I tests for screening of substances for potential as endocrine disruptors. The Phase I studies have contributed to the initial portion of this optimization. These factors will be used for the rest of the optimization in Part II. Without these initial studies we would not have used the best gaseous atmosphere for optimal testosterone concentrations from the testicular tissues.

7.0 CONCLUSIONS

The testosterone RIA and the LDH assay can both be verified with M-199 without phenol red. Both were validated and show the characteristics necessary for use to optimize the sliced testis assay.

There were certain factors in the initial Phase I experiments that definitely were not beneficial to use in the assay, for instance, 22 week old rats do not show the responsiveness in their testicular tissue that is necessary for an optimal assay. The air atmosphere was also not a favorable condition for the assay. The prototypical assay media, Media 199 without phenol red, was equal to any of the others tested. Statistical analysis was necessary to show that the atmosphere of 5% CO_2/ 95% O_2 was optimal and that rats of the 11-15 week range could be used for the assay. From these conclusions , we were ready to advance to the Phase II experiments.

Media 199 without phenol red will be used after it is gassed with the 5% CO_2/ 95% O_2 mixture and pH adjusted to 7.4 for testicular tissues from 11-15 week old rats for the Phase II experimental studies.

8.0 REFERENCES

EP Evaluator, release 3.0 statistical analysis software from David Rhoads Associates, Inc., Kennett Square, PA.

FQPA (1996). Food Quality Protection Act of 1996, U.S. Public Law 104-170, 21 U.S.C. 46a(p), Section 408(p), 110 STAT.1489, August 3, 1996.

APPENDIX 1

LHD Validation and Verification with Media 199 without Phenol Red